Increased apoptosis in the heart of genetic hypertension, associated with increased fibroblasts

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Abstract

Objective: The present studies were undertaken to identify apoptosis in cardiomyocytes of genetic hypertension and to study the relationship among apoptosis, aging and blood pressure, and the effect of angiotensin-converting enzyme (ACE) inhibitors on apoptosis.

Methods: Apoptosis in the hearts of spontaneously hypertensive rats (SHR) was identified by electron microscopy (EM) and DNA laddering, and quantified from age 3 weeks to 64 weeks in comparison with normotensive rats (WKY). Fibroblasts and protein products of Bcl-2 and Bax were measured by quantitative immunohistochemistry. SHR were treated with ramipril, an ACE inhibitor. Results: The results showed that: (1) ultrastructural characteristics of apoptosis were observed in cardiomyocytes of SHR, with shrinkage of the cell and condensation of the cytoplasm and chromatin. A DNA ladder was shown; (2) a significant increase in apoptosis in SHR began as early as age 4 weeks and reached a plateau at 16 weeks and maintained at high levels up to 64 weeks. Blood pressure (BP) in SHR started to increase significantly at age 5 weeks; (3) fibroblasts were significantly increased in the heart of SHR; (4) the ratio of Bcl-2/Bax was significantly reduced in SHR; and (6) ramipril effectively reduced apoptosis and fibroblasts, and increased the ratio of Bcl-2/Bax.

Conclusion: Apoptosis occurs in the cardiomyocytes of genetic hypertension although fibroblasts are increased, and a significant, age-dependent increase in apoptosis is observed. The increase in apoptosis occurs before the difference in blood pressure is detectable. The ACE inhibitor ramipril may be useful for prevention of apoptosis in the heart.

Keywords: Angiotensin; Apoptosis; Extracellular matrix; Fibrosis; Hypertension; Myocytes

1. Introduction

Apoptosis or program cell death is a gene-controlled cell suicide [1,2]. The role of apoptosis in diverse physiological processes and in a variety of pathological conditions has received considerable attention in recent years [1–3]. Apoptosis in heart and vasculature has recently been recognized.

Recently, some investigators have suggested that apoptosis may play a key role in heart disease, particularly heart failure [4]. The fundamental issue in heart failure is what causes the progressive loss of cardiomyocytes in a chronic process. As a death process induced by internal signalling triggered by imbalance in the biological conditions, apoptosis could be the cause for the loss of cardiomyocytes.

There are a variety of pathological conditions that eventually lead to heart failure. Hypertension is one of the very important conditions. Hypertension can result in diastolic and systolic heart failure. However, how this clinical setting eventually results in heart failure is unknown. We hypothesise that apoptosis could be involved in the mechanism that causes the progressive loss of cardiomyocytes in the failing heart of hypertension.

The most characteristic features of apoptosis are the morphological changes recognisable by electron microscopy (EM) and the internucleosomal cleavage of genomic DNA into fragments that are discrete multiples of 185–200 bp that produce a typical DNA ladder after agarose gel electrophoresis (DNA laddering) [1,2]. Ultrastructural characteristics of apoptosis, e.g. condensation and margination of chromatin and shrinkage of the cell, which can only be clearly seen under EM, have been demonstrated in

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certain cells, mostly tumor cells. However, the ultrastructural morphology of apoptosis in cardiomyocytes of a hypertensive heart has not been reported. A recent study on infarct hearts with EM [5] has shown that the previous claimed ‘apoptotic’ myocytes in the infarct area of hearts may be oncotic myocytes with DNA fragmentation. That study highlights the importance of using EM for studies on apoptosis [6]. Since the apoptotic morphology remains the gold standard for identification of apoptosis, it is necessary to investigate this issue in the cardiomyocytes of a hypertensive heart.

Angiotensin-converting enzyme (ACE) inhibitors have been routinely used to treat heart failure and hypertension. Prevention of cardiovascular remodelling has been proposed as the key mechanism of the efficacy of these drugs [7]. However, the precise mechanism of how these drugs prevent cardiovascular remodelling and could also be involved in cardiovascular remodelling in pathological conditions. We hypothesise that prevention of apoptosis could be one of the mechanisms for the efficacy of ACE inhibitors. Ramipril, an ACE inhibitor, has a long-acting effect, can be used once daily and is welcomed by clinicians and patients. Whether ramipril has an effect on apoptosis is unknown.

In the present studies, we used spontaneous hypertensive rat (SHR) as a model (a genetic hypertensive model) to study the following issues: (1) Does apoptosis occur in the genetic hypertensive heart? (2) If yes, how is it related to the age and the development of hypertension? (3) Can the rate of apoptosis be changed by the ACE inhibitor ramipril? We use the spontaneously hypertensive model as an approach to study the issue of apoptosis in hypertension, because over 90% of patients with hypertension have spontaneous hypertension (or essential hypertension). These studies may also provide necessary information for our future studies on other models of hypertension.

2. Methods

2.1. Animals and reagents

Rats (72 male SHR and 60 WKY) were obtained from the animal house of Austin Hospital, Heidelberg, Australia. Ethic approval for using these animals has been granted by Animal Ethic Committee of Austin Hospital. BP was measured by the indirect tail-cuff technique (WandW recorder, model 8005) in preheated, lightly restrained rats. Hearts were obtained from SHR and WKY at different ages (age 3, 4, 6, 8, 16, 32 and 64 weeks). The hearts were isolated, put into liquid nitrogen immediately and stored at −70°C. Ramipril, a gift from Hoechst Marion Roussel Pharmaceutical, was used to treat SHR, with untreated SHR and WKY as controls. Young SHR (n=6) were treated with ramipril (1 mg/kg, once daily), and the control SHR (n=6) and WKY (n=6) with distilled water, from 3 to 10 weeks of age. The other reagents were from Sigma Chemical (St Louis, MO) unless otherwise stated.

2.2. Transmission electron microscopy (EM)

Preparation of cardiac tissues of SHR or WKY was performed as previously described [8]. Briefly, portions of cardiac tissues from SHR or WKY were fixed in 2.5% glutaraldehyde in 0.1 M PBS at 4°C overnight. The fixed tissues were washed with distilled water, dehydrated through graded acetone, and embedded in Araldite-Epon resin (CIBI -GEIGY, Lane Cove, NSW, Australia). Thin sections were cut and stained with saturated uranyl nitrate and Reynolds lead citrate, and viewed in a JEOL-1200 EX electron microscope (JEOL, Tokyo, Japan).

2.3. DNA laddering

DNA extraction and electrophoresis of α32PdCTP-labelled DNA fragments (DNA laddering) was performed as previously described [8]. A 10-μl α32PdCTP labelled (see below) DNA sample (about 100 ng) was precipitated for 2 h in −20°C ethanol containing 0.3 M sodium acetate and 0.01 M MgCl2 (final concentration) and centrifuged for 30 min, 4°C, at 12 000 g. The precipitated, labelled DNA was washed twice with 70% cold ethanol and unincorporated α32PdCTP removed. The labelled DNA was resuspended in TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA), mixed with 5 ml of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% ficoll) and electrophoresed for 3 h at 5.0 v/cm in 1.0% agarose gel using 0.5X TBE buffer (45 mM Tris-borate, 1 mM EDTA) as running buffer. The gel was removed and dried in a Gel-Drying Frame (Kem-En-Tec, Haraldsgade, Denmark) following gel equilibration with 3% of glycerol for 1 h. Dried gel was sealed in plastic wrap and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY) with an intensifying screen for 2–3 h at −70°C.

2.4. Saturation labelling assay

Saturation labeling of the 3’ end of apoptotic DNA fragmentations from the cardiac tissues of SHR and WKY was performed as previously described [8]. A 20-μl reaction mixture containing 4 μl of 5X buffer (Boehringer–Mannheim, Germany), 2 μl of 2.5 mM CoCl2, 1000 ng DNA, and increasing concentrations of ddCTP (Pharmacia, Uppsala, Sweden) and α32PdCTP (Bresatec). The ratio of ddCTP:α32PdCTP was 4.7:1 per reaction. (The increasing concentrations in pmol were 1.93:0.41, 3.85:0.82, 7.76:1.65, 15.5:3.3, 31.0:6.6, and 62.0:13.2 each in triplicate). After taking 2 μl as background, 20 U TdT (Pharmacia, Uppsala, Sweden) were added. The reaction was proceeded for 60 min at 37°C, and stopped by adding 2 μl of 0.5 M EDTA. Detection of the α32PdCTP...
L-droxlase of fibroblasts, which is a specific cell component of the nucleolmma, and contained large sharply defined chromatin masses (arrow). Clumped ill-defined nucleoplasm (arrow). A normal cardiac myocyte obtained from WKY was used as control. The nuclei of the nuclei (N) in the normal cell (Fig. 1a, c) showed even distribution of chromatin and a normal nucleolus (n). An apoptotic cell from SHR illustrates early ultrastructural changes of apoptosis (Fig. 1b). The nucleus (N) showed shrinkage characterized by the appearance of a clear gap around the nucleus and contained small marginalized electron dense chromatin masses (arrow). Clumped ill-defined nuclear chromatin was also present (arrowhead). Another cell (Fig. 1c) manifests further advanced apoptosis compared with the cardiomyocyte in Fig. 1b. 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marginated electron dense chromatin masses that abutted the nuclear envelope as well as large clumped ill-defined nuclear chromatin. These results identify that the cardiomyocytes of SHR undergo apoptosis. Apoptosis in SHR was also shown by the electrophoresis of DNA ladder pattern that showed a typical nucleosome ladder in cardiac tissue obtained from SHR (Fig. 2, lane 1,3).

3.2. Quantification of DNA fragments in the cardiac tissues of SHR

DNA fragments of the cardiac tissues in SHR and WKY were quantitated at age 3, 4, 6, 8, 16, 32 and 64 weeks. The results showed that there was a significant age-dependent increase in apoptosis in SHR, and the labeling of DNA breaks in SHR started to increase significantly at age 4 weeks ($L_{\text{max}}$: 12.34±0.94 pmol/mg DNA in SHR vs. 9.97±0.78 in WKY, $P<0.05$) and reached a plateau at 16 weeks (37.90±2.84 pmol/mg DNA) (Fig. 3a). The values were maintained at high levels up to 64 weeks (39.35±2.5 in SHR vs. 11.44±1.78 in WKY, $n=6$, $P<0.01$). When the DNA from WKY was tested, there was no significant increase in the labeling of DNA breaks with increasing age, although the trend showed a slight gradual increase in apoptosis from age 3 weeks to 64 weeks (the values of $L_{\text{max}}$ were 9.90±0.81 pmol/mg DNA at 3 weeks vs. 11.44±1.78 at 64 weeks, $n=6$, $P>0.1$) (Fig. 3a). Meanwhile, the blood pressure of these SHR started to increase significantly at age 5 weeks, and reached a plateau at 16 weeks (Fig. 3b). Interestingly, the increase in DNA fragmentation in SHR was earlier than the occurrence of the significant increase in BP.
3.3. Fibroblasts in the hearts

The results showed that the quantity of fibroblasts was significantly increased in the hearts of SHR (P<0.01, n=6), compared to those of WKY (Fig. 4). Treatment of SHR with ramipril significantly reduced the quantity of fibroblasts compared with untreated SHR (P<0.01, n=6) (Fig. 4).

3.4. Bcl-2 and Bax in the hearts

Bcl-2 proteins were significantly lower in SHR than in WKY (P<0.01, n=6, Fig. 5). In contrast, Bax proteins were significantly higher in SHR than in WKY (P<0.01, n=6, Fig. 5).

3.5. Effect of ACE inhibitor on apoptosis

The DNA fragmentation of SHR treated with ramipril was significantly reduced (Lmax, 12.94±2.30 pmol/mg DNA) compared to untreated SHR (Lmax, 27.20±2.30 pmol/mg DNA) (P<0.01) (Fig. 6), while there was no significant difference in the DNA fragmentation between the SHR treated with ramipril and WKY control (Lmax, n=6, Fig. 5). Ramipril increased the quantity of Bcl-2 proteins and reduced that of Bax (P<0.01, n=6, Fig. 5).
There are five main findings in the present studies: (1) ultrastructural characteristics of apoptosis in the cardiomyocyte of SHR were identified by EM and apoptosis in SHR was also shown by the biochemical method DNA ladder pattern; (2) an age-dependent significant increase in apoptosis occurred in SHR from age 4 weeks, reached a plateau at age 16 weeks and maintained at high levels up to 64 weeks; (3) the increase in apoptosis appeared earlier (age 4 weeks) than did the increase in blood pressure (5 weeks); (4) The quantity of fibroblasts was significantly increased in the heart of SHR; (5) the ratio of Bcl-2/Bax was significantly reduced in SHR; and (6) ramipril effectively reduced apoptosis and fibroblasts, and increased the ratio of Bcl-2/Bax.

We have revealed a typical morphology of apoptosis in the cardiomyocytes of SHR. The results clearly demonstrate that apoptosis occurs in the cardiomyocytes of SHR. Since we have detected apoptosis in SHR by four different methods (electron microscopy, DNA laddering, quantification of DNA fragmentation, and quantification of apoptotic regulatory proteins), we feel confident that apoptosis does occur in genetic hypertension.

By quantification, the present studies demonstrate the time course for the development of apoptosis, hypertension and age. It indicates clearly that apoptosis is increased in genetic hypertension and is associated with aging. The studies also demonstrate that the increase in apoptosis occurs earlier (4 weeks) than does BP (5 weeks). There are two explanations for this phenomenon. One is the tail-cuff method for measurement of BP may not be sensitive enough to detect the subtle increase in BP in the early life of SHR in which an increase in apoptosis has been detected. The other explanation is that the triggering of apoptosis may not depend on BP but on other factors such as neurohumoral factors that also cause hypertension. Studies by other investigators have shown that angiotensin II (AlI), a blood pressure producer, can cause apoptosis in cultured cardiomyocytes [11]. This direct evidence suggests that apoptosis in SHR might not, or at least not solely, depend on blood pressure.

One question is in which cell types did the increased apoptosis occur? The heart consists of many cell types. Besides cardiomyocytes, fibroblasts are another major group of cells in heart. It could be possible that the increased apoptosis is caused by an increase in apoptosis of fibroblasts. However, in the present studies the quantity of the fibroblasts was actually increased but not reduced in SHR. We believe that the increased apoptosis most likely occurs in cardiomyocytes, which has actually been observed in our EM results. However, the possibility of increased apoptosis in fibroblasts cannot be completely ruled out, because apoptosis and mitosis in fibroblasts may both increase but the balance between them may be in favour of mitosis in this case.

The progressive loss of cardiomyocytes in the heart may play a key role in pathogenesis of heart failure (including diastolic and systolic failure). It is well known that adult cardiomyocytes do not undergo mitosis. Therefore, the lost cardiomyocytes cannot be re-grown, but can be replaced by other cells such as fibroblasts (In fact, our results did show an increase in fibroblasts in SHR). Studies [12] have been reported that cardiomyocytes are gradually lost in aging rat heart, which is accompanied with a gradual increase in fibrous tissues, although the cardiomyocyte volume is increased (hypertrophy) in the aging heart. The morphologic changes result in elevated left ventricular
end-diastolic pressure and decreased dP/dt [12], which indicates that a significant impairment of ventricular function occurs with senescence. In our studies, there was a gradual slight increase in apoptosis with aging in WKY. Although the increase is not statistically significant it does show the trend (to 64 weeks only). We believe that a significant increase in apoptosis in the pathological conditions of hypertension speeds up the process to heart failure. Therefore, to reduce apoptosis in the heart could become a therapeutic target for future treatment of hypertension and heart failure.

Our present studies showed that the treatment of genetic hypertension with ramipril was associated with a reduction in apoptosis and a prevention of an increase in fibroblasts. Our results also showed that ramipril interfered a signal transduction pathway of apoptosis: the regulatory proteins Bcl-2 and Bax. The protein product of oncogene Bcl-2 is anti-apoptotic and that of Bax is pro-apoptotic. Their activity appears to control the cell death pathway at a checkpoint between signals from the cell surface and activation of the effector proteases [13]. It is proposed that reduction of the ratio of Bcl-2/Bax activates apoptosis. Direct evidence that AngII stimulates apoptosis in cultured cardiomyocyte has been reported [11]. Taken these together, it is likely that the mechanism by which ACE inhibitors improve heart failure may be at least in part due to prevention of apoptosis in cardiomyocytes, which prevents cardiac remodelling.

One argument is that reduction of hypertrophy of the heart may be achieved by stimulation of apoptosis with a pharmacuetic agent. We believe that may not be true in cardiomyocytes, because adult cardiomyocytes do not undergo mitosis and the hypertrophy is caused by enlargement of the cell volume. Stimulation of apoptosis can only reduce the number of the existing cardiomyocytes, which may do more harm than good because the progressive loss of cardiomyocytes is the fundamental problem that leads to heart failure.

In conclusion, the present studies demonstrate that apoptosis may be involved in the pathogenesis of genetic hypertension and heart failure. The inhibition of apoptosis in the heart may be one of the mechanisms of ACE inhibitors to prevent cardiac remodelling in hypertension and heart failure.

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References